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Exopolysaccharides Produced by Intestinal *Bifidobacterium* Strains Act as Fermentable Substrates for Human Intestinal Bacteria[▽]

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Eleven exopolysaccharides (EPS) isolated from different human intestinal *Bifidobacterium* strains were tested in fecal slurry batch cultures and compared with glucose and the prebiotic inulin for their abilities to act as fermentable substrates for intestinal bacteria. During incubation, the increases in levels of short-chain fatty acids (SCFA) were considerably more pronounced in cultures with EPS, glucose, and inulin than in controls without carbohydrates added, indicating that the substrates assayed were fermented by intestinal bacteria. Shifts in molar proportions of SCFA during incubation with EPS and inulin caused a decrease in the acetic acid-to-propionic acid ratio, a possible indicator of the hypolipidemic effect of prebiotics, with the lowest values for this parameter being obtained for EPS from the species *Bifidobacterium longum* and from *Bifidobacterium pseudocatenulatum* strain C52. This behavior was contrary to that found with glucose, a carbohydrate not considered to be a prebiotic and for which a clear increase of this ratio was obtained during incubation. Quantitative real-time PCR showed that EPS exerted a moderate bifidogenic effect, which was comparable to that of inulin for some polymers but which was lower than that found for glucose. PCR-denaturing gradient gel electrophoresis of 16S rRNA gene fragments using universal primers was employed to analyze microbial groups other than bifidobacteria. Changes in banding patterns during incubation with EPS indicated microbial rearrangements of *Bacteroides* and *Escherichia coli* relatives. Moreover, the use of EPS from *B. pseudocatenulatum* in fecal cultures from some individuals accounted for the prevalence of *Desulfovibrio* and *Faecalibacterium prausnitzii*, whereas incubation with EPS from *B. longum* supported populations close to *Anaerostipes*, *Prevotella*, and/or *Oscillospira*. Thus, EPS synthesized by intestinal bifidobacteria could act as fermentable substrates for microorganisms in the human gut environment, modifying interactions among intestinal populations.

The human colon is inhabited by a complex bacterial community dominated by obligate anaerobes that reach counts over 10^{11} bacteria per gram of content. Colonic bacteria obtain energy by fermenting a variety of dietary compounds that are not digested by the host. The fermentation of nondigestible compounds from the diet, which include mainly plant cell wall polysaccharides and some storage polysaccharides and oligosaccharides, has a major influence on host health (6). Short-chain fatty acids (SCFA) are the main products arising from the microbial fermentation of these carbohydrates, and they exert health benefits by providing energy to the colonic epithelium, modulating some metabolic processes, suppressing pathogenic bacteria in the intestine, and exerting immune stimulation (7, 42). Specific carbohydrates are now being widely used as prebiotic substrates based on the concept that they stimulate particular beneficial bacteria (19) and that, at the same time, are able to reduce the populations of nonutilizing and nonbeneficial bacteria by competition. Inulin and fructo-oligosaccharides (FOS) were originally proposed to be prebiotics that selectively stimulate bifidobacteria, intestinal microorganisms considered to be beneficial and that are being extensively used as probiotics (31). Experimental evidence confirmed the bifidogenic effect of inulin and FOS (18, 28, 49). However, additional studies using molecular techniques have revealed that a variety of other bacterial groups, including *Clostridium*-related species, also respond to inulin or FOS (13, 26). Among the possible explanations for the diversity in response to prebiotics is that complex gut microbial communities involve extensive metabolic interactions. Metabolic products produced from dietary prebiotics by one bacterial species may provide substrates to support the growth of other populations. Such a mechanism, termed "cross-feeding," can result in metabolic consequences that would not be predicted simply from the substrate preferences of isolated groups of bacteria. These interactions may lead to changes in bacterial communities within the colon following pH variations or the release of fermentation and other metabolic products formed by the bacterial metabolic activity (2, 15, 16, 39, 47).

Many strains of lactic acid bacteria and bifidobacteria are able to produce exopolysaccharides (EPS). The physiological functions of these carbohydrate polymers have not yet been clearly determined. It has been suggested that EPS produced by some lactic acid bacteria could exert beneficial effects on human health. Among these effects, the possibility of acting as prebiotic substrates has been demonstrated successfully to date by Korakli and coworkers (27) for a fructan-type EPS produced by one strain of *Lactobacillus sanfranciscensis*. There was evidence of a bifidogenic effect for the levan-type EPS

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TABLE 1. Identification by partial sequencing the 16S rRNA V1-V2 gene region of EPS-producing *Bifidobacterium* strains of human intestinal origin employed in this study

Species	Strain	Origin	GenBank accession no.
B. animalis	C64MR	Rectal mucosa	EU430030
	E43	Feces	EU430031
B. pseudocatenulatum	A102	Feces	EU430027
	C52	Feces	EU430029
	E63	Feces	EU430025
	E515	Feces	EU430026
	H34G	Feces	EU430028
B. longum	E44	Feces	EU430035
	H67	Feces	EU430032
	H73	Feces	EU430033
	L55	Feces	EU430034

produced by another strain of the same species (8). We recently found that some human intestinal *Bifidobacterium* isolates were able to produce EPS and that some of them harbored genes relating to the synthesis of heteropolysaccharides (36). In the present work, we have investigated the abilities of some of these EPS synthesized by intestinal bifidobacteria to act as fermentable substrates for microorganisms inhabiting the human colon as well as their potential bifidogenic effects.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The EPS-producing strains (Table 1) employed in this study were previously isolated from biological samples of 10 healthy adult volunteers with the approval of the Regional Ethics Committee (Asturias, Spain) and were identified by 16S rRNA gene sequencing. Volunteers mainly provided fecal samples, and some of them also provided colonic or rectal mucosal samples that were obtained during routine colonoscopies (10). Bifidobacteria were grown in MRSC broth (MRS broth [BioKar Diagnostics, Beauvais, France] supplemented with 0.25% [wt/vol] L-cysteine [Sigma Chemical Co., St. Louis, MO]) and incubated at 37°C for 24 h under conditions of anaerobiosis (10% [vol/vol] H_2, 10% CO_2, and 80% N_2) in a Mac 500 anaerobic chamber (Don Whitley Scientific, West Yorkshire, United Kingdom). After centrifugation, microorganisms were resuspended in MRSC broth containing 20% glycerol and kept at −80°C until use. Prior to performing experiments, the correct identities of strains were corroborated by 16S rRNA gene sequencing (Table 1).

EPS isolation. The EPS fraction of each strain was isolated from the cellular biomass harvested from agar-MRSC plates (35). Strains were grown by creating a culture lawn with sterile glass beads on the surface of agar-MRSC plates that were then incubated under anaerobic conditions at 37°C for 5 days. Cell biomass was collected using 2 ml of ultrapure water per plate, and the resulting volume was mixed with 1 volume of 2 M NaOH. The suspension was stirred overnight at room temperature to favor the release of the polymer from the cellular surface. Cells were removed by centrifugation, and EPS was precipitated from the supernatants during 48 h at 4°C using 2 volumes of cold absolute ethanol. After centrifugation, the EPS fraction was resuspended in ultrapure water and dialyzed for 3 days at 4°C with daily changes of water using dialysis tubes (Sigma) with a 12- to 14-kDa molecular mass cutoff. Finally, the dialyzed EPS fractions were freeze-dried.

Fecal batch culture fermentations. Three independent fecal batch fermentations, each of them corresponding to samples from three different donors, were carried out in the carbohydrate-free basal medium (CFBM) previously described by Al-Tamimi and coworkers (1). Briefly, the medium contained peptone water (2 g/liter), yeast extract (2 g/liter), sodium chloride (0.1 g/liter), dipotassium phosphate (0.04 g/liter), magnesium sulfate (0.01 g/liter), hexahydrated calcium chloride (0.01 g/liter), monosodium carbonate (2 g/liter), L-cysteine (2.5 g/liter), bile bovine (0.5 g/liter), Tween 80 (2 ml), hemin (50 mg/ml), and vitamin K (10 μl).

For each batch, CFBM was distributed into different glass tubes (2.5 ml per tube) along with 15 mg of the EPS fractions isolated from 11 *Bifidobacterium* strains (Table 1), glucose, or inulin (Sigma). One additional tube was kept without adding carbon source and was used as a control. After the complete dissolution of the carbon sources and sterilization (120°C for 15 min), the tubes were maintained overnight under conditions of anaerobiosis at 37°C before use.

The fecal slurry inocula were prepared as follows: feces from three healthy adults (two females and one male, 25 to 37 years old) who had not recently received antibiotics were separately diluted 1/10 in sterile 0.17 M phosphate-buffered saline (pH 7.3) and homogenized with a Lab-Blender 400 stomacher (Seward Medical, London, United Kingdom) for 2 min. The fecal homogenates of each donor (10 ml) were poured into 90 ml of CFBM and allowed to stabilize by being kept overnight at 37°C under anaerobic conditions. The control and tubes containing the different EPS fractions, glucose, or inulin were mixed with 2.5 ml of the stabilized fecal slurry. Fermentations were carried out under anaerobic conditions at 37°C for 5 days. Samples were taken at time zero, 24 h, and 5 days. Eight hundred microliters of cultures was centrifuged (12,000 × *g* for 10 min) each time, and pellets and supernatants were collected.

Determination of pH in controls at time zero and after 5 days of incubation was carried out by using pH indicator strips (Merck, Darmstadt, Germany) according to the manufacturer's instructions.

Analysis of SCFA in fecal batch cultures by gas chromatography-mass spectrometry (MS). Cell-free supernatants from fecal batch cultures were filtered through 0.2-μm filters, mixed with a 1/10 mixture of ethyl-butyric acid (2 mg/ml) as an internal standard, and stored at −80°C until analysis.

A system composed of a 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) connected to a mass spectrometry (MS) 5973N detector (Agilent) was used to quantify the SCFA. Data were collected with Enhanced ChemStation G1701DA software (Agilent). Samples (1 μl), prepared as described above, were directly injected into the gas chromatograph equipped with an HP-Innowax capillary column (60-mm length by 0.25-mm internal diameter, with a 0.25-μm film thickness; Agilent) using He as the gas carrier, with a constant flow rate of 1.5 ml/min. The temperature of the injector was kept at 220°C, and the split ratio was 50:1. Chromatographic conditions were as follows: an initial oven temperature of 120°C, 5°C/min up to 180°C, 1 min at 180°C, and 20°C/min up to 220°C for cleaning the column. The column was directly connected to the MS detector, and the electron impact energy was set to 70 eV. The data collected were in the range of 25 to 250 atomic mass units (at 3.25 scans/s). SCFA were identified by comparing their mass spectra with those held in the HP-Wiley 138 library (Agilent) and by comparing their retention times with those of the corresponding standards (Sigma). The peaks were quantified as the relative total ionic count abundance with respect to the internal standard. The concentration (mM) of each SCFA was calculated using linear regression equations ($R^2 \geq 0.99$) from the corresponding curves of standards obtained with six different concentrations. Total SCFA concentrations were calculated as the sum of the three major SCFA (acetic acid plus propionic acid plus butyric acid). The molar proportion of each SCFA was obtained as the concentration percentage with respect to the total SCFA.

DNA extraction. DNA was extracted from pellets harvested from 800 μl of fecal batch cultures. Cells were washed once in phosphate-buffered saline, and DNA was extracted with the QIAamp DNA stool kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples were stored at −20°C until use.

Analysis of bifidobacteria by quantitative real-time PCR. The quantification of the *Bifidobacterium* population in fecal batch cultures was performed by quantitative real-time PCR using previously described genus-specific primers (20).

All reactions were performed using MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA), and amplifications were carried out using a 7500 Fast Real Time PCR system (Applied Biosystems) with Sybr green PCR master mix (Applied Biosystems). One microliter of purified DNA was used as the template in the 25-μl PCR mixture. Thermal cycling consisted of an initial cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves were made with *Bifidobacterium longum* strain NCIMB8809, which was grown overnight in MRSC broth under anaerobic conditions. Standard curves were obtained by plotting the threshold cycle values obtained for the standard culture as a linear function of the base-10 logarithm of the initial number of cells in the culture determined by plate counting. The number of *Bifidobacterium* cells in fecal samples was determined by comparing the threshold cycle values obtained to the standard curve. Samples were analyzed in duplicate in at least two independent PCR runs.

Analysis of fecal microbiota by PCR-DGGE. The evolution of the intestinal microbiota during fecal batch fermentations was analyzed by partial amplifica-

TABLE 2. Molar concentrations and proportions of the three major SCFA in fecal cultures from three donors using glucose, inulin, or EPS from bifidobacteria as carbon sources

Carbon source	Strain	Time (days)	Mean concn (mM) ± SD				Proportion (%) ± SD			Mean acetic acid/propionic acid ratio ± SD
			Acetate	Propionate	Butyrate	Total SCFA	Acetate	Propionate	Butyrate	
Control (without carbohydrate added)		0	4.03 ± 1.65	0.96 ± 0.36	1.20 ± 0.37	6.18 ± 2.36	64.51 ± 2.44	15.56 ± 1.50	19.93 ± 2.09	4.18 ± 0.46
		1	8.50 ± 0.57	2.27 ± 0.26	3.03 ± 0.48	13.80 ± 1.07	61.64 ± 2.19	16.51 ± 1.73	21.86 ± 1.93	3.77 ± 0.46
		5	8.27 ± 1.42	1.90 ± 0.61	2.27 ± 0.71	12.44 ± 1.67	66.53 ± 7.18	14.97 ± 3.19	18.50 ± 5.85	4.68 ± 1.32
EPS isolated from *B. animalis*	C64MR	1	15.63 ± 5.75	4.77 ± 1.50	4.24 ± 1.27	24.64 ± 8.38	63.02 ± 3.29	19.54 ± 1.79	17.43 ± 1.68	3.26 ± 0.44
		5	13.79 ± 1.94	3.99 ± 0.95	3.79 ± 1.36	21.57 ± 3.61	64.40 ± 6.26	18.35 ± 2.16	17.25 ± 4.45	3.60 ± 0.87
	E43	1	17.96 ± 7.11	5.40 ± 1.93	4.63 ± 1.61	27.99 ± 10.60	63.85 ± 1.86	19.44 ± 0.80	16.71 ± 1.16	3.29 ± 0.22
		5	14.89 ± 3.14	4.68 ± 0.93	4.44 ± 1.64	24.01 ± 5.16	62.23 ± 5.74	19.59 ± 1.84	18.18 ± 4.44	3.23 ± 0.62
EPS isolated from *B. pseudocatenulatum*	A102	1	12.95 ± 3.84	4.08 ± 1.56	3.94 ± 1.24	20.98 ± 6.59	62.03 ± 2.36	19.12 ± 1.33	18.85 ± 1.61	3.26 ± 0.33
		5	13.22 ± 2.22	5.01 ± 0.77	4.69 ± 0.87	22.92 ± 3.58	57.65 ± 3.13	21.88 ± 1.21	20.47 ± 2.51	2.65 ± 0.26
	C52	1	17.25 ± 6.17	6.13 ± 1.89	5.28 ± 1.51	28.66 ± 9.30	59.89 ± 4.35	21.46 ± 1.74	18.65 ± 2.67	2.82 ± 0.44
		5	14.24 ± 3.74	5.99 ± 1.04	5.99 ± 1.19	26.22 ± 5.86	53.91 ± 3.03	23.07 ± 1.26	23.02 ± 1.99	2.35 ± 0.25
	E63	1	14.01 ± 5.49	4.49 ± 1.41	4.14 ± 1.52	22.63 ± 8.35	61.50 ± 2.87	20.21 ± 2.23	18.29 ± 0.85	3.09 ± 0.45
		5	11.39 ± 1.60	4.03 ± 1.17	3.54 ± 1.05	18.96 ± 2.99	60.80 ± 5.98	20.91 ± 3.47	18.29 ± 3.01	3.01 ± 0.73
	E515	1	14.30 ± 2.92	4.56 ± 1.02	4.79 ± 1.40	23.65 ± 5.25	60.72 ± 1.88	19.28 ± 0.70	20.01 ± 1.68	3.16 ± 0.18
		5	12.87 ± 2.88	4.70 ± 1.40	4.52 ± 1.29	22.09 ± 5.01	58.49 ± 5.53	21.01 ± 1.80	20.49 ± 4.52	2.82 ± 0.49
	H34G	1	13.50 ± 4.88	4.44 ± 1.72	4.18 ± 1.49	22.12 ± 8.06	61.00 ± 1.76	19.98 ± 0.73	19.02 ± 1.28	3.06 ± 0.19
		5	10.99 ± 2.10	3.62 ± 1.26	3.92 ± 1.59	18.54 ± 4.65	60.75 ± 7.55	19.07 ± 3.26	20.18 ± 4.75	3.33 ± 0.96
EPS isolated from *B. longum*	E44	1	14.57 ± 4.20	5.22 ± 1.47	4.36 ± 1.20	24.15 ± 6.69	60.28 ± 2.28	21.68 ± 2.57	18.04 ± 1.37	2.83 ± 0.48
		5	15.00 ± 3.50	6.73 ± 1.85	5.04 ± 1.67	26.81 ± 6.87	56.35 ± 3.61	25.14 ± 1.81	18.51 ± 2.08	2.26 ± 0.31
	H67	1	11.98 ± 1.73	3.57 ± 0.53	3.74 ± 0.78	19.29 ± 2.89	62.20 ± 2.59	18.54 ± 1.50	19.26 ± 1.46	3.38 ± 0.40
		5	12.15 ± 3.56	5.81 ± 1.40	4.04 ± 1.61	22.01 ± 6.03	55.04 ± 2.90	26.64 ± 1.74	18.31 ± 1.44	2.08 ± 0.24
	H73	1	12.81 ± 2.96	3.70 ± 0.89	3.78 ± 1.10	20.29 ± 4.85	63.25 ± 2.52	18.30 ± 1.99	18.44 ± 1.17	3.50 ± 0.45
		5	12.33 ± 2.29	4.99 ± 1.38	4.00 ± 1.28	21.32 ± 4.84	58.46 ± 4.34	23.13 ± 1.73	18.41 ± 2.61	2.56 ± 0.43
	L55	1	11.38 ± 3.51	3.48 ± 1.07	3.67 ± 1.06	18.53 ± 5.50	61.24 ± 4.04	18.86 ± 2.52	19.90 ± 1.58	3.33 ± 0.67
		5	11.76 ± 2.33	5.39 ± 1.18	4.93 ± 0.88	22.08 ± 4.09	53.23 ± 3.56	24.29 ± 1.15	22.47 ± 2.87	2.20 ± 0.24
Glucose		1	14.45 ± 3.90	2.73 ± 0.53	2.48 ± 0.82	19.65 ± 4.14	72.89 ± 6.65	13.97 ± 1.56	13.13 ± 5.15	5.32 ± 1.05
		5	14.66 ± 2.75	2.42 ± 0.44	3.32 ± 2.58	20.41 ± 2.28	72.15 ± 12.19	11.84 ± 1.54	16.01 ± 11.37	6.26 ± 1.69
Inulin		1	14.36 ± 0.39	5.18 ± 1.24	4.57 ± 2.16	24.10 ± 2.76	60.22 ± 6.70	21.40 ± 4.36	18.39 ± 6.64	2.95 ± 0.84
		5	14.44 ± 4.33	5.14 ± 1.70	5.16 ± 2.63	24.74 ± 3.41	57.64 ± 13.55	20.45 ± 4.48	21.91 ± 12.86	3.02 ± 1.26

tion of the 16S rRNA gene using universal primers. The PCR products were detected by denaturing gradient gel electrophoresis (DGGE), and specific DNA bands were selected by taking into account differences observed among EPS and individuals. Bands were identified by sequencing and comparison with those sequences held in the GenBank database.

Primers 357F (5′-TAC GGG AGG CAG CAG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) were used to amplify the V3 variable region of the 16S rRNA gene (32). An additional GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG-3′) was also added to primer 357F to obtain primer 357F-GC. Amplifications were performed using an iCycler apparatus (Bio-Rad Laboratories, Hercules, CA). The reaction mixture (50 μl) contained 0.25 μM each primers 357F-GC and 518R, 200 μM each deoxynucleoside triphosphate (Amersham Bioscience, Uppsala, Sweden), 2.5 U *Taq* polymerase (Eppendorf, Hamburg, Germany), and 3 μl of DNA. The amplification program was as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 40 s; and a final extension step at 68°C for 10 min. The PCR products were separated by DGGE in a DCode system (Rio-Rad) on 8% (vol/vol) polyacrylamide (37.5:1 acrylamide-bisacrylamide; Bio-Rad) gels (dimensions, 200 by 200 by 1 mm) containing a 40% to 60% gradient of urea-formamide in Tris-acetate-EDTA (TAE) buffer (50× TAE is 2 M Tris, 1 M acetic acid, and 50 mM EDTA [pH 8.0]). The 40% gradient solution contained 20 ml acrylamide-bisacrylamide, 2 ml 50× TAE, 16 ml formamide, and 18.8 g urea; the 60% gradient solution contained 20 ml acrylamide-bisacrylamide, 2 ml 50× TAE, 24 ml formamide, and 25.2 g urea. Seventeen milliliters of each solution was mixed with 153 μl of 10% (wt/vol) ammonium persulfate and 15.3 μl *N,N,N′,N″*-tetramethylethylenediamine. Gels were made with a 475 gradient delivery system (Bio-Rad). Electrophoresis was performed at 85 V in TAE (1×) buffer at a constant temperature of 60°C during 16 h. The gels were stained with ethidium bromide for 30 min, washed with ultrapure water, and visualized and photographed under UV light using a Gel Doc 2000 system with Quantity One software (Bio-Rad).

Bands of DNA were excised from gels with a plastic tip, poured into 50 μl ultrapure water, and kept overnight at 4°C. These DNA samples were used to perform secondary PCR amplifications using primers 357F (without a GC clamp) and 518R under the conditions indicated above. The PCR products were purified using the GenElute PCR cleanup kit (Sigma). Automated sequencing of one strand of PCR products was done at Secugen S.L. (Madrid, Spain) with primer 518R in an ABI Prism gene sequencer (Applied Biosystems, Foster City, CA). The Basic Local Alignment Search Tool (BLAST) program was used to assess the identities of the sequences obtained with those held in the GenBank database.

RESULTS

EPS isolation. EPS fractions were isolated from *Bifidobacterium* strains grown on MRSC plates instead of a liquid medium in order to avoid the coisolation of glucomannans present in the yeast extract added to MRS (45). The level of production was low and ranged from 0.78 to 4.34 mg per plate depending on the strain, with a protein content in fractions between 1.9% and 8.9% (data not shown).

Evolution of SCFA in fecal batch cultures. Fecal batch cultures with added EPS displayed pH values between 6 and 7 from the beginning to the fifth day of incubation (data not shown). However, cultures with glucose and inulin that showed initial pH values between 6 and 7 decreased to pH values between 4 and 5 or 5 and 6 after 5 days of incubation, respectively.

There was evidence of differences among the individuals with respect to the levels of SCFA attained in fecal cultures, with individual 2 being the highest producer for all EPS tested but not for inulin and glucose (data not shown). However, we used the mean data from donors for subsequent comparisons in order to facilitate the analyses (Table 2).

Total SCFA levels increased during the first 24 h of incubation of fecal microbiota with and without external carbon sources added (Table 2). Acetic acid was the most abundant, followed by similar amounts of both propionic acid and butyric acid. Minor amounts of other acids such as isobutyric, isovaleric, valeric, and caproic acids were also identified (data not shown). However, the increase in SCFA levels was considerably more pronounced in the presence of glucose, inulin, and EPS than in the control, thus indicating a stimulation of SCFA production from the external carbon sources added. During incubation, the highest level of production of total SCFA was obtained with *Bifidobacterium pseudocatenulatum* C52, *B. longum* E44, and *Bifidobacterium animalis* E43, which indicates that SCFA production was probably more related with the polymer composition than with the producer strain.

In general, the molar proportion of acetic acid decreased during incubation with EPS and inulin but increased during incubation with glucose. The contrary occurred for propionic acid, which increased during incubation, while butyric acid increased or decreased moderately depending on the compound (Table 2). Different fermentation patterns were thus seen according to the carbohydrate used. The highest molar proportion of propionic acid was obtained for the EPS isolated from the four strains of the species *B. longum*, followed by *B. pseudocatenulatum* strain C52. On the fifth day of fermentation, these five polymers clearly promoted higher molar proportions of propionic acid than inulin. The molar proportion of acetic acid in cultures with glucose was higher, and those of propionic and butyric acids were lower, than in cultures with inulin and EPS. Remarkably, as a consequence of the SCFA production pattern mentioned above, the acetic acid-to-propionic acid ratio decreased during incubation with the EPS tested and inulin, whereas a clear increase in this ratio was found in cultures with glucose. In this respect, it is worth noting that the lowest values for the acetic acid/propionic acid ratio (values around 2 on the fifth day of incubation, which were lower than those found for inulin) were obtained with EPS isolated from strains of the species *B. longum* (E44, H67, H73, and L55) and from *B. pseudocatenulatum* C52.

Changes in *Bifidobacterium* populations analyzed by quantitative real-time PCR. Quantitative real-time PCR analyses were performed to evaluate the bifidogenic effect of the EPS isolated from intestinal bifidobacteria (Fig. 1). After 1 or 5 days of incubation, all EPS tested, as well as glucose and inulin, promoted higher increases in levels of bifidobacterium populations than those occurring in control cultures without carbohydrates added, which was indicative of a stimulatory effect of these substrates on bifidobacteria. The increase in the *Bifidobacterium* population promoted specifically by EPS was in the range of that promoted by inulin. In general, the highest stimulation after 1 day of incubation was promoted by glucose, followed by inulin and EPS from *B. pseudocatenulatum* C52, although a great interindividual variability was found. After 5 days of incubation, the strongest stimulation corresponded again to glucose followed by inulin and different EPS depending on the individual. The lowest initial count of bifidobacteria in fecal samples generally resulted in the highest increase in counts after incubation.

Changes in fecal microbial cultures analyzed by PCR-DGGE. PCR-DGGE was used as a molecular technique to monitor major qualitative changes in the compositions of microbial groups in fecal batch cultures (Fig. 2). Subsequent amplification and sequencing of several bands from DGGE gels allowed the identification of several microbial groups present in the cultures (Table 3), some of which were related with moderate differences in profiles seen through incubation. The most noticeable changes found in cultures during incubation are summarized in Table 4. Figure 2a shows DGGE banding patterns of control cultures without carbohydrate added in feces from two individuals, since the behavior in samples from the third individual followed a pattern similar to those of the others (data not shown). Initial differences between donors and moderate variations after 5 days of incubation were found. These differences were related mainly to the disappearance or attenuation of several upper bands (bands 2, 3, 7, 8, and 9) that matched with different *Bacteroides* species. Therefore, changes occurring in the same way as those mentioned above were inherent to interindividual variability and to the methodology used and were not considered to be related to the carbon sources added. In spite of that, in cultures with glucose, band 3 remained clearly visible throughout incubation, which indicates that glucose positively contributed to the survival of some microorganisms related to *Bacteroides vulgatus*.

Moderate differences in DGGE banding patterns among

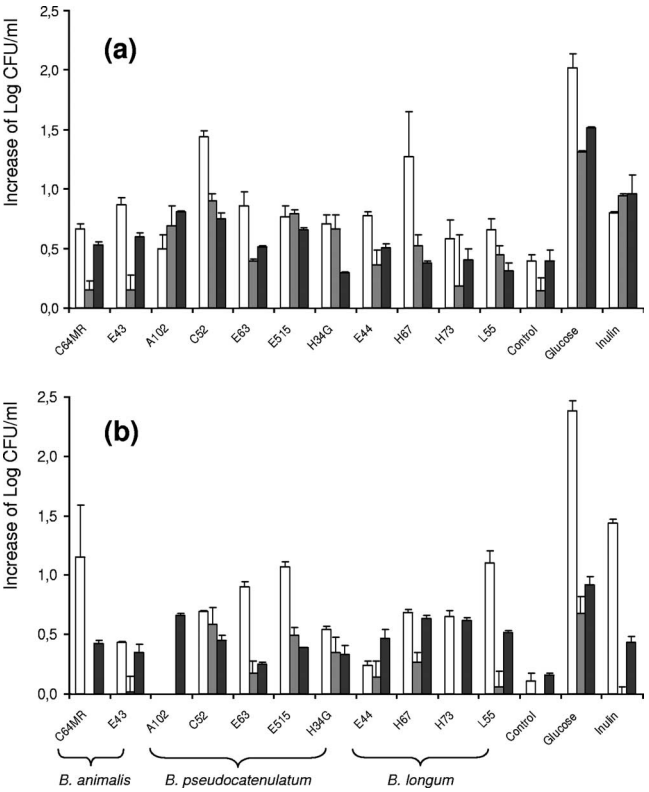

FIG. 1. Increase with respect to time zero of *Bifidobacterium* counts measured by quantitative real-time PCR in fecal slurry cultures from three donors using glucose, inulin, or EPS isolated from intestinal bifidobacteria as carbon sources after 24 h (a) and 5 days (b) of incubation. The control does not include carbohydrate source added. Initial *Bifidobacterium* counts are as follows: 9.56 ± 0.10 log CFU/ml for donor 1 (white bars), 10.61 ± 0.14 log CFU/ml for donor 2 (gray bars), and 9.93 ± 0.08 log CFU/ml for donor 3 (black bars).

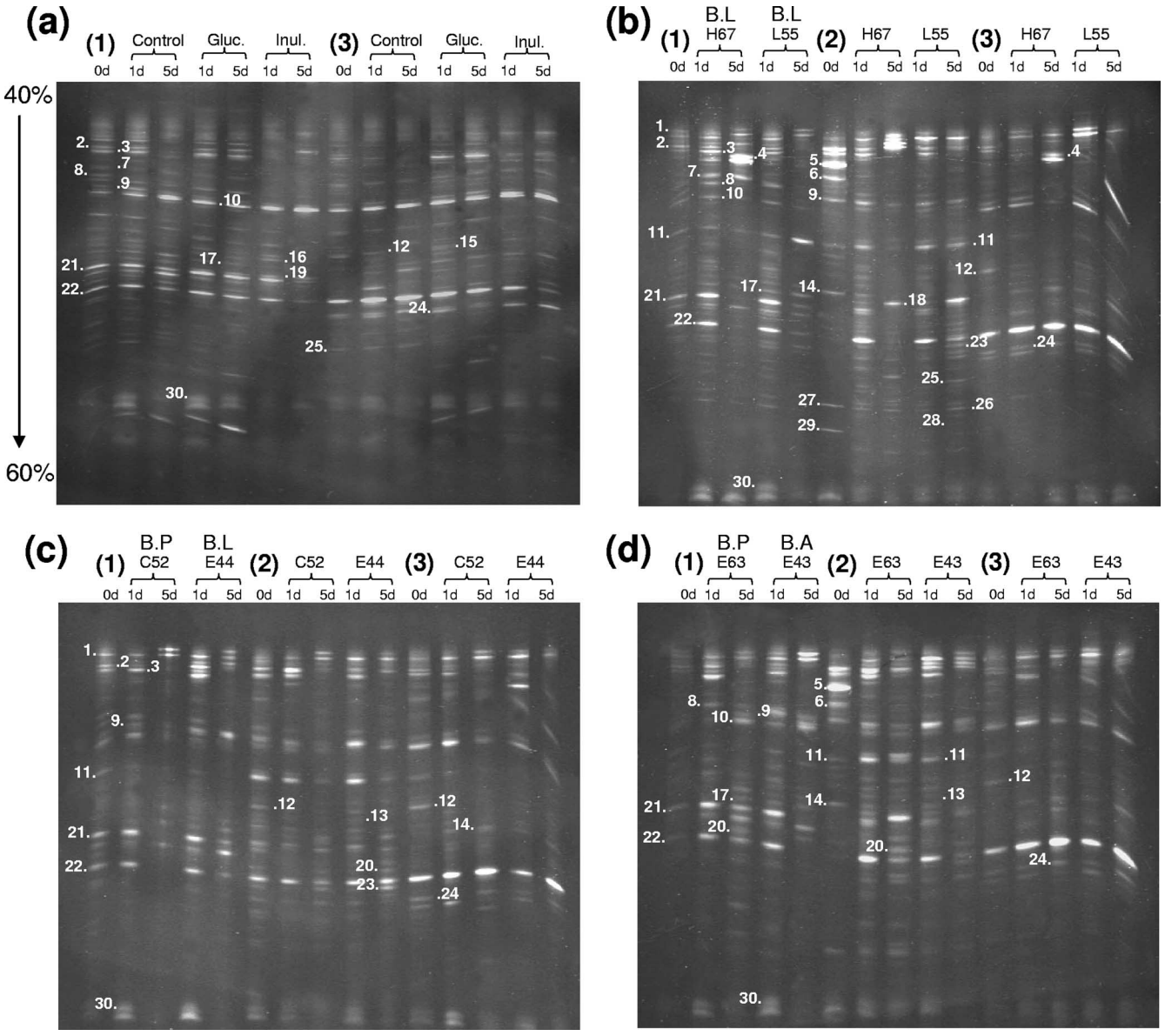

FIG. 2. DGGE profiles of fecal cultures from three donors using glucose, inulin, or EPS isolated from intestinal bifidobacteria as carbon sources at time zero and after 1 and 5 days of incubation. Numbers (1, 2, and 3) in parentheses indicate different donors. (a) Control culture without carbohydrate added. Gluc., glucose; Inul., inulin. (b) EPS isolated from *B. longum* H67 and *B. longum* L55. (c) EPS isolated from *B. pseudocatenulatum* C52 and *B. longum* E44. (d) EPS isolated from *B. pseudocatenulatum* E63 and *B. animalis* E43. 0d corresponds to the initial microbiota of each donor before incubation. Numbers inside gels refer to sequenced DNA bands, whose amplicons and closest relatives are indicated in Table 3. BL, *B. longum*; BP, *B. pseudocatenulatum*; BA, *B. animalis*.

fecal batch cultures with EPS were found throughout incubation (Fig. 2b, c, and d and Table 4). These differences were related mainly to different fingerprintings of feces among different donors on the first day of incubation and to a reduction in band diversity on the fifth day. Most clear and EPS-specific noticeable changes are commented on below.

In the presence of polymers from *B. longum* strains H67 and L55 (Fig. 2b), band 18, which matched *Anaerostipes caccae* and/or *Clostridium polysaccharolyticum*, appeared to be reinforced in fecal cultures of individual 2. One band, marked as band 4, also became more intense in fecal cultures from two donors (donors 1 and 3) during incubation with H67, and this band displayed homology with *Prevotella copri*. The same occurred for band 11 in fecal cultures from individuals 1 and 2 with EPS L55, and in this case, the band matched *Oscillospira guilliermondii*. Band 12, also related to *O. guilliermondii*, weakened in feces from donors 2 and 3 with the EPS from *B. longum* E44 (Fig. 2c), whereas band 23, which was closely related to *Oscillibacter valericigenes*, became enriched in feces from individual 2 during incubation with the same polysaccharide from E44. In fecal cultures from some donors, incubation with the three EPS from *B. longum* strains tested (H67, L55, and E44) promoted an initial increase in intensity of bands 21 and/or 22 related to *Escherichia coli* and/or *Shigella flexneri*, but the in-

TABLE 3. Bacteria identified by sequencing of DGGE bands amplified by PCR from DNA of fecal batch cultures using universal 16S rRNA gene-targeted primers 357F and 518R

Band[a]	Closest sequence found in GenBank database (% similarity)[b]	GenBank accession no. of closest sequence	Closest known species (% similarity)[b]	GenBank accession no. of closest known species
1	Uncultured bacterium clone SJTU C02 96 (91)	EF403967	*Bacteroides uniformis* (90)	AB247146
2	Uncultured bacterium clone L243 (94)	AY983015	*Bacteroides vulgatus* (94)	EU136687
3	Uncultured *Bacteroidales* bacterium clone MS104A1 (97)	EF703593	*Bacteroides vulgatus* (97)	EU136687
4	Uncultured bacterium clone SSH 51 (100)	EU268853	*Prevotella copri* (96)	AB244773
5	Uncultured *Bacteroidales* bacterium clone MS146A1 B01 (99)	EF706714	*Bacteroides vulgatus* (99)	CP000139
6	Uncultured bacterium clone LZ51 (97)	AY976451	*Prevotella copri* (94)	AB244772
7	Uncultured bacterium clone SSH 65 (90)	EU268866	*Bacteroides plebeius* (90)	AB200217
8	Uncultured *Bacteroidales* bacterium clone pigH8 (92)	AY695697	*Bacteroides thetaiotamicron*/*Bacteroides acidofaciens* (91)	AY895189/AB021164
9	*Bacteroides uniformis* strain JCM5828 (93)	EU136680		
10	*Bacteroides uniformis* strain JCM5828 (99)	EU136680		
11	Uncultured *Lachnospiraceae* bacterium clone MS069A1 F08 (96)	EF706465	*Oscillospira guilliermondii* (96)	AB040499
12	Uncultured *Lachnospiraceae* bacterium clone MS069A1 F08 (96)	EF706465	*Oscillospira guilliermondii* (97)	AB040499
13	Uncultured *Lachnospiraceae* bacterium clone MS146A1 E12 (93)	EF706759	*Clostridium coccoides* (92)	EF025906
14	*Clostridium symbiosium* strain 69 (99)	EF025909		
15	Uncultured bacterium clone RL181 aan97c06 (100)	DQ798481	*Ruminococcus gauvreaui*/*Coprococcus comes* (100)	EF529620/EF031542
16	Uncultured bacterium clone NA 35 (100)	AY975545	*Coprococcus catus* (100)	ABO38359
17	Uncultured bacterium clone RL185 aan87f05 (100)	DQ825275	*Faecalibacterium prausnitzii* (100)	AJ413954
18	Uncultured *Ruminococcus* sp. isolate, DGGE gel band 51v3 (98)	EF587944	*Anaerostipes caccae*/*Clostridium polysaccharolyticum* (98)	AB243986/X77839
19	*Ruminococcus* sp. strain WAL 14507 (100)	EF036467	*Peptostreptococcus productus*/*Ruminococcus obeum* (100)	AM117591/AY169419
20	Uncultured bacterium clone SJTU C0806 (99)	EF404297	*Desulfovibrio piger* (98)	AF192152
21	Uncultured bacterium clone isolate, DGGE gel band wj22 (97)	EF489276	*Escherichia coli*/*Shigella flexneri* (97)	CP000800/CP000266
22	Uncultured bacterium clone isolate, DGGE gel band wj22 (100)	EF489276	*Escherichia coli*/*Shigella flexneri* (98)	EU330541/CP000266
23	Uncultured bacterium clone SJTU C06 05 (100)	EF404193	*Oscillibacter valericigenes* (97)	AB238598
24	Uncultured *Lachnospiraceae* bacterium clone MS135A1 G06 (98)	EF706692	*Clostridium orbiscindens*/*Eubacterium plautii* (97)	AY730665/AY724678
25	Uncultured bacterium clone API13S48 (93)	AM277960	*Clostridium leptum* (96)	AJ305238
26	*Citrobacter freundii* strain SW3/*Kluyvera ascorbata* strain CDC 0648/74 (98)	EU124385/ AF176560		
27	Uncultured bacterium clone RL 188 aan96f11 (100)	DQ802797	*Dialister invisus* (99)	AY162469
28	Uncultured *Lachnospiraceae* bacterium clone MS146A1 D07 (100)	EF706742	*Clostridium orbiscindens*/*Eubacterium plautii* (100)	AY730665/AY724678
29	*Dialister invisus* (96)	AY162469		
30	Uncultured bacterium G3 clone 24 (100)	EF149169	*Escherichia coli*/*Shigella flexneri* (99)	AM779083/CP000266

[a] See Fig. 2 for bands.
[b] Accession numbers were determined by searching the GenBank database using the local BLAST program. We selected those sequences showing maximum identity among those displaying maximum coverage when aligned with sequences from PCR-DGGE amplicons as closest relatives.

tensity of one or both bands decreased notably after 5 days of incubation. Major bands found after 5 days of incubation of fecal samples with EPS isolated from *B. longum* strains included members of *Bacteroides* and/or *Prevotella*, *E. coli*-related bacteria, *Oscillospira* and/or *Oscillibacter*, and *Anaerostipes* and/or *Clostridium polysaccharolyticum*.

With respect to the EPS from *B. animalis* strain E43 (Fig. 2d), a decrease in intensity of bands 21, 22, and 30 (related to

TABLE 4. Main PCR-DGGE bands showing changes (variation of intensity, appearance, or disappearance) in fecal cultures from three donors during incubation with several EPS from bifidobacteria used as carbon sources

Carbon source	Strain	Band(s) showing change		
		Individual 1	Individual 2	Individual 3
Control (without carbohydrate added)		2, 3, 7, 8, 9		2, 3, 7, 8, 9
EPS from *B. longum*	H67	4, 21, 22	18, 21, 22	4
	L55	11, 21, 22	11, 18, 21, 22	
	E44	21, 22	12, 23	12
EPS from *B. animalis*	E43	9, 10, 21, 22, 30	9, 10	9
EPS from *B. pseudocatenulatum*	C52	11, 21, 22	11, 21, 22	11
	E63	17, 20, 21, 22	11, 20, 21, 22	

E. coli and/or *S. flexneri*) was seen in fecal cultures from individual 1 at the end of incubation. Changes in intensity (increases or decreases) of bands 9 and 10, matching with *Bacteroides uniformis*, were also found, depending on individuals. Nevertheless, major bands identified after 5 days of incubation corresponded to microorganisms of the genus *Bacteroides* and *E. coli* and related microorganisms.

Finally, EPS from *B. pseudocatenulatum* C52 and E63 (Fig. 2c and d) were related to increases or decreases in the intensity of bands 21 and 22 matching with *E. coli* and/or *S. flexneri*. Remarkably, during incubation with the E63 EPS, band 17, identified as being *Faecalibacterium prausnitzii* (100% identity), and band 20, sharing homology with the species *Desulfovibrio piger*, became slightly more intense in fecal cultures from individual 1 and from individuals 1 and 2, respectively. With C52 EPS, band 11, related to *O. guilliermondii*, decreased in intensity during incubation in fecal cultures of the three individuals, whereas with E63 EPS, it remained clearly visible during the incubation of feces from donor 2. At the end of incubation, major bands in cultures of EPS from *B. pseudocatenulatum* were related to the group of *Bacteroides*, *E. coli*, *F. prausnitzii*, *Desulfovibrio*, and *Oscillospira*.

DISCUSSION

Genetic studies (36) and preliminary analyses carried out by us on the composition of EPS from human intestinal bifidobacteria indicated that they are heteropolysaccharides containing glucose, galactose, and, in several cases, rhamnose. The low level of production of EPS obtained for most intestinal bifidobacteria is in agreement with that previously reported by other authors, who indicated that heteropolysaccharides are produced in considerably lower amounts than homopolysaccharides (5, 44).

We observed the effects of EPS isolated from bifidobacteria in terms of metabolic activity and composition of the microbial community in fecal slurry cultures. First, all carbohydrates used seemed to act as fermentable substrates for the intestinal microbiota, clearly enhancing the production of SCFA during incubation. Levels of SCFA attained in fecal cultures with EPS were comparable to that obtained with the prebiotic inulin. Similarly to that previously indicated by other workers, incubation with glucose gave rise to more acetic acid but less propionic and butyric acid production than other fermentable substrates (34, 46). The lower pH values found by us in cultures with glucose and inulin, with respect to the cultures with EPS, were probably due to a greater stimulation of growth and/or metabolic activity of intestinal bacteria by the former substrates. However, a shift to butyrate production was not evident in these mostly acidic cultures with glucose and inulin, as indicated to occur by Walker et al. (47) when the pH of fecal cultures decreased to values lower than 6. Molar proportions of the different SCFA measured in our fecal cultures were within the same order of that reported by others. Thus, most authors indicated that acetic acid is produced at higher levels than propionic and butyric acids (1, 4, 9, 25, 46). Incubation of the intestinal microbiota in the presence of our EPS or inulin promoted a shift in the SCFA profile of fecal cultures, causing a decrease through the incubation of molar proportions of acetic acid, an increase in propionic acid levels, decreases or moderate increases in butyric acid levels, and a reduction in the acetic acid-to-propionic acid ratio through time. This behavior was contrary to that found in cultures with glucose, a carbohydrate not considered to be prebiotic and for which a clear increase in the acetic acid-to-propionic acid ratio was obtained through fermentation. Increases in total SCFA production with shifts toward propionic and butyric acids were previously reported for inulin in a simulator of the human intestinal microbial ecosystem (43). The reduction in the acetic acid-to-propionic acid ratio has been proposed as a possible indicator of the hypolipidemic effect (inhibition of cholesterol and fatty acids biosynthesis in liver, which finally results in a decrease in lipid levels in blood) of prebiotics (11). Remarkably, the highest decrease in this ratio was obtained by us for the four cultures with EPS from the species *B. longum* and with the EPS C52 from *B. pseudocatenulatum*. Interestingly, values for the acetic acid/propionic acid ratio of these five EPS were even lower than those obtained by us for the prebiotic inulin. However, although EPS producer microorganisms were isolated from different human individuals (10) and in spite of the different percentages of monosaccharides in their compositions (data not shown), the identical chemical nature of these polymers cannot be absolutely discarded.

The bifidogenic effect of EPS was shown by real-time quantitative PCR analyses that showed a moderate increase in levels of bifidobacteria during incubation of the fecal slurry cultures. The degree of stimulation depended on the EPS and the donor and was lower than that obtained with glucose, being comparable in general to that found with inulin. Bifidobacteria have been described to be beneficially affected by inulin and inulin-derived substrates (24, 49). The stimulation of the intestinal *Bifidobacterium* population was shown previously for an oat-based product cofermented with a β-glucan-producing *Pediococcus damnosus* strain (30). A bifidogenic effect was also demonstrated for a levan-type and a fructan-type EPS produced by two strains of *Lactobacillus sanfranciscensis* (8, 27) of food origin. However, the bifidogenic effect of EPS produced by intestinal bifidobacteria has not previously been reported. Nevertheless, since bifidobacteria are acetate and lactate producers, beneficial shifts in SCFA profiles found by us in fecal cultures point not only toward a unique bifidogenic effect but also to the modification of other microbial groups that may use acetate and produce propionate or butyrate via a metabolic cross-feeding mechanism (2, 14, 15).

PCR-DGGE is a useful technique for the examination of the diversity within microbial communities. In fecal slurry cultures, the use of universal primers allows the monitoring of some microbial groups and the ability to obtain an overview of major qualitative changes affecting them. We were not able to identify bifidobacteria with this technique, probably due to a limitation of the universal primers and experimental conditions used in this work. The use of EPS isolated from intestinal bifidobacteria as a carbon source gave rise to a reduction in the variability of bands and to an enrichment of some microbial populations other than bifidobacteria, as previously found by Dal Bello et al. (8) with the EPS produced by *L. sanfranciscensis*. All amplicons sequenced in our study showed homology with members of the indigenous gut microbiota previously found by other authors (21, 41, 48). Major groups represented included members of *Bacteroides*, *Clostridium leptum* (cluster

IV), *Clostridium coccoides* (cluster XIVa), and gammaproteobacteria. In our case, global changes in cultures with EPS affected mainly *Bacteroides* and *E. coli* and relatives. Thus, sequencing of the DNA fragment bands pointed to a reduction in levels of several populations of *E. coli* and/or *S. flexneri* after incubation with polymers from *B. longum* and *B. animalis*. For polymers from *B. pseudocatenulatum*, increases or decreases in the intensities of several bands matching with *E. coli* and/or *S. flexneri* were found, suggesting a rearrangement of populations from this group. Some other interesting features were also found with EPS from *B. pseudocatenulatum* and *B. longum*. Regarding the polymers from the first species, a moderate increase in the intensity of bands corresponding to *Desulfovibrio* and *F. prausnitzii* was found during incubation. *F. prausnitzii* is an established member of the dominant human fecal microbiota that is able to produce butyrate, lactate, and formate and that needs acetate in the medium for growth (12). In addition, it has been demonstrated that *Desulfovibrio* had an important role in the turnover of SCFA in the colon (17). Chemostat culture experiments corroborated these findings and indicated that sulfate-reducing bacteria like *Desulfovibrio* and others promoted a shift in the metabolism of sugars, altering the synthesis by saccharolytic microorganisms of hydrolytic enzymes involved in carbohydrate breakdown (33). It is thus possible that the high SCFA level, low acetic acid/propionic acid ratio, and high bifidogenic effect obtained with the EPS produced by *B. pseudocatenulatum* strain C52 could be related to a positive interaction between this polymer, saccharolytic microorganisms related to *Bacteroides* or other groups, and sulfate-reducing populations from fecal cultures. With respect to EPS from *B. longum*, during incubation, they seemed to support populations of *Anaerostipes*, *Prevotella*, and/or *Oscillospira*, depending on the polymer used. *Oscillospira* is a microorganism that has not yet been grown in pure culture. It is found in the rumen of several animals (29), and it has also been recovered from the human large intestine by using 16S rRNA gene clone libraries (21). Members of the genus *Prevotella*, as happens with its neighbor *Bacteroides*, are saccharolytic versatile microorganisms that are able to utilize a wide variety of carbohydrates as fermentable carbon sources (23, 37, 38). Under the appropriate conditions, *Prevotella* is able to produce propionate (40). Traditionally isolated from ruminal material, several strains of this genus were recently recovered from human feces and proposed as a new species (22). *Anaerostipes* is a common inhabitant of the human gut; it is an efficient lactate converter (14) and is able to produce butyrate from lactate formed by bifidobacteria in cocultures (2). Metabolic cross-feeding among different members of the colon microbiota was suggested to be a possible mechanism responsible for colonic butyrate and propionate production (2, 3, 14). Two types of mechanisms of cross-feeding between *Bifidobacterium* and butyrate-forming bacteria have recently been described, one due to the consumption of fermentation end products (lactate and acetate) and the other due to the cross-feeding of partial breakdown products formed from complex carbohydrates (2).

Interactions among acetate or lactate formers, poly- or oligosaccharide degraders, and butyrate or propionate producers or even more complex relationships such as those that can occur between sulfate-reducing bacteria and other microorganisms can be established among intestinal microbial populations as affected by the carbon sources available. These interactions promoted by microbial EPS could have accounted for in vitro shifts in SCFA profiles, increases in levels of bifidobacteria, and changes in intestinal microbial patterns of fecal cultures found in the present work. Sugars such as glucose are not abundant in the gut, but other complex polymers present in the diet, such as inulin, can reach this location, or even others, such as microbial EPS, may be synthesized in this environment. Therefore, it is reasonable to suppose that EPS from intestinal bifidobacteria could act as fermentable substrates in vivo, promoting shifts in SCFA profiles and changes in relationships among intestinal microbial populations. In this way, a WHO expert group has recently recommended to revise the definition of prebiotics by not only considering the effect on bifidobacteria and lactobacilli but also broadening it to other ecological interactions among members of the human microbiota (50).

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